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Physiology

Genetic separation of tumor growth and hemorrhagic phenotypes in an estrogen-induced tumor

(angiogenesis/cancer)

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ABSTRACT **Chronic administration of estrogen to the Fischer 344 (F344) rat induces growth of large, hemorrhagic pituitary tumors. Ten weeks of diethylstilbestrol (DES) treatment caused female F344 rat pituitaries to grow to an average of 109.2 ± 6.3 mg (mean ± SE) versus 11.3 ± 1.4 mg for untreated rats, and to become highly hemorrhagic. The same DES treatment produced no significant growth (8.9 ± 0.5 mg for treated females versus 8.7 ± 1.1 for untreated females) or morphological changes in Brown Norway (BN) rat pituitaries. An F_1 hybrid of F344 and BN exhibited significant pituitary growth after 10 weeks of DES treatment with an average mass of 26.3 ± 0.7 mg compared with 8.6 ± 0.9 mg for untreated rats. Surprisingly, the F_1 hybrid tumors were not hemorrhagic and had hemoglobin content and outward appearance identical to that of BN. Expression of both growth and morphological changes is due to multiple genes. However, while DES-induced pituitary growth exhibited quantitative, additive inheritance, the hemorrhagic phenotype exhibited recessive, epistatic inheritance. Only 5 of the 160 F_2 pituitaries exhibited the hemorrhagic phenotype; 36 of the 160 F_2 pituitaries were in the F344 range of mass, but 31 of these were not hemorrhagic, indicating that the hemorrhagic phenotype is not merely a consequence of extensive growth. The hemorrhagic F_2 pituitaries were all among the most massive, indicating that some of the genes regulate both phenotypes.**

Tumor growth, angiogenesis, and tissue invasion are all normal biological processes that have escaped normal physiological control. Thus, control of tumors requires the understanding of both the regulation of cell cycle progression and the regulation of morphological changes such as degradation of basement membranes by secreted proteases (1–3). Genetic analysis is a powerful tool to delineate such complex regulatory pathways. The estrogen-induced rat pituitary tumor is a useful model system for this purpose.

Chronic treatment of Fischer 344 (F344) rats with estrogen [either estradiol or the synthetic estrogen diethylstilbestrol (DES)] induces hypertrophy and hyperplasia of the lactotroph cells of the pituitary (4–6). A great increase in cell proliferation is evidenced by increased DNA synthesis rate and increased DNA content (6, 7). These pituitary tumors undergo qualitative and quantitative changes in blood supply (6, 8, 9). While a normal pituitary receives much of its blood supply via a portal system from the hypothalamus, the estrogen-induced pituitary tumor is invaded by arteries from the systemic blood supply (8). Histological analysis has revealed estrogen-dependent breakdown of basement membrane in the F344 rat pituitary (9). After several weeks of continuous DES treatment, the F344 rat pituitary is 5 to 10 times normal mass with drastic morphological changes and internal hemorrhagic lakes (6, 9, 10).

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Unlike F344 rats, rat strains such as Holtzman and Sprague–Dawley are able to control their pituitary response to estrogen (7, 10, 11). Holtzman rat pituitaries exhibit increased DNA synthesis during the first 2 to 4 days of estrogen treatment, but then return to unstimulated rates despite prolonged estrogen treatment (7). Holtzman rat pituitaries do sustain increased prolactin synthesis in response to prolonged estrogen treatment (10). This indicates that their lack of tumor formation is not due to a general unresponsiveness to estrogen but, rather, is due to genetic variation in the ability to control estrogen-dependent growth and development.

The existence of rat strains differing greatly in their ability to control their pituitary response to estrogen allows the use of genetic analysis to study the mechanism of this tumor growth *in vivo*. However, the genetics of the estrogen-induced pituitary tumor is complex. Previous analysis of the segregation of pituitary tumor formation in crosses of F344 and Holtzman rats has indicated that tumor formation is a polygenic trait due to variation at two or three as yet unidentified genetic loci (12).

We present evidence, obtained from segregation of tumor phenotypes in simple genetic crosses between F344 rats and the tumor-resistant Brown Norway (BN) rat strain, that extensive estrogen-dependent cell proliferation of the pituitary is separable from the hemorrhagic phenotype. Our data indicate that the F344 pituitary tumor is due to multiple genes as proposed previously (12). However, while tumor growth behaves as an additive trait, the hemorrhagic phenotype is expressed as a recessive epistatic trait. Tumor phenotypes in progeny of an F_2 intercross indicate that a subset of the genes regulating estrogen-dependent tumor growth also regulate morphological changes but by a different mechanism.

MATERIALS AND METHODS

Animals. Rats of the strains F344/NHsd (F344), BN/SsNHsd (BN), BUF/NHsd (Buffalo), COP/Hsd (Copenhagen 2331), WKY/NHsd (Wistar Kyoto), and HsdHot:Holtzman SD (Holtzman) were obtained from Harlan–Sprague–Dawley. F344 and BN rats were crossed in our facility to produce the F_1 hybrid. F_1 siblings were mated to produce the F_2. All rats were weaned at 19 days of age.

DES Treatment. Rats (21 days of age) were given subcutaneous implants of 5 mg crystalline DES encapsulated in Silastic tubing (Dow Corning) while under light ether anesthesia (12). Untreated animals were of the same age but were not given an implant. During the treatment period, animals were fed Lab Diet brand rat diet 5012 (PMI Feeds, St. Louis) and water ad libitum and kept on a 12-hour light/12-hour dark cycle. At the end of the treatment period, animals were decapitated and the pituitaries removed. The hemorrhagic phenotype was scored by visual inspection. The pituitaries were immediately weighed and frozen on dry ice; pituitaries were stored at −70°C until use.

Abbreviations: DES, diethylstilbestrol; F344, Fischer 344 rats; BN, Brown Norway rats.

Biochemical Assays. Frozen pituitaries were thawed, blotted briefly on a buffer saturated filter, and homogenized individually in 40 mM potassium phosphate buffer, pH 7.5, with 10 strokes of a glass-Teflon homogenizer. Homogenates were immediately frozen in liquid nitrogen and stored at −70°C. Hemoglobin was assayed by its pseudoperoxidase activity with chlorpromazine (Sigma) as a substrate (13). DNA was assayed by the diphenylamine method (14). Each assay on each animal was performed in triplicate, which were averaged to give the value for that animal. Six DES-treated and four untreated animals of each genotype were assayed for both hemoglobin and DNA. Their data were averaged to give the value for each genotype and treatment.

Statistics. Significance testing to compare DES-treated to untreated rats within genotypes (see Fig. 2) was done by a one-sided *t* test. Statistical comparison of hemoglobin levels of F_2 pituitaries to the F_1 mean (see Fig. 3*C*) was done using a two-sided *t* test.

Mass was expressed on a log scale to bring distributions closer to normal distribution because variance of pituitary mass within genotypes exhibits a scale effect with the mean (15). When group statistics were calculated for the log of pituitary mass, individual animal data were first transformed and then statistics, such as mean and variance, were calculated.

RESULTS

Tumor-Resistant Inbred Strain. The tumor-resistant rats used in most other studies, Holtzman and Sprague–Dawley, are outbred strains (8, 11, 12). F344 is an inbred strain and, for our genetic experiments, we desired to also use an inbred strain as a tumor-resistant control to minimize within-strain phenotypic variation. A small fraction of Holtzman rats exhibit significantly increased pituitary mass (12). Although two other inbred strains, ACI (11) and Wistar Furth (16, 17), are susceptible to estrogen-induced pituitary tumors, no definite resistant inbred strains have been reported, though experiments by Dunning *et al.* (18) suggest that the Copenhagen 2331 strain may be resistant to estrogen induction of pituitary tumor over the short term. To find a tumor-resistant inbred rat strain, we tested an array of strains for their response to 8 weeks of DES treatment. The strains Copenhagen 2331, BN, Buffalo, and Wistar Kyoto were chosen for analysis because they are commercially available, lack known characteristics that would interfere with the analysis of estrogen-induced tumors, and are independently derived from outbred progenitors (19).

Of all of the strains tested, none underwent a highly significant increase in pituitary mass, except for F344 (Table 1). Holtzman, Buffalo, and Copenhagen 2331 experienced small increases in pituitary mass of marginal statistical significance (Table 1). The minuscule mass increases exhibited by BN and Wistar Kyoto were statistically insignificant (Table 1). We chose BN as our tumor-resistant control in further experiments. Our initial data (Table 1) and further examination (Table 2) indicate pituitaries of DES-treated BN rats are less variable in mass than the outbred Holtzman strain (12).

Table 2. Effect of 10 weeks of DES treatment on pituitary mass in female rats

		Mean mass		Variance of mass	
	n	mg	$\log_{10}$(mg)	[mg]^2	[log10(mg)]^2
BN, DES	16	8.9	0.94	3.5	0.009
BN, untreated	6	8.7	0.92	7.1	0.018
F344, DES	25	109.0	2.02	999.0	0.020
F344, untreated	4	11.3	1.04	7.6	0.012
F_1, DES	29	26.3	1.41	13.0	0.004
F_1, untreated	5	8.6	0.92	4.3	0.012
F_2, DES	160	32.1	1.46	292.8	0.034

We examined pituitary mass on both a linear and log scale. Log_{10} transformation of pituitary mass was used because pituitary mass exhibits a scale effect with the mean. In the data of Wiklund *et al.* (12), variance of pituitary mass in three genetic groups of rats, Holtzman, F344, and their F_1 hybrid, increased with group mean, even though the rats were given the same 8 week DES treatment. For example, DES-treated Holtzman females had a mean pituitary mass of 12.3 mg and a variance of 20.3, whereas F344 females had a mean of 88.0 mg and a variance of 745.3 (12). Our present data also exhibit this trend (Table 2). This phenomenon is known as a scale effect and can be compensated for by transformation of the data such as $\log_{10}$ (15).

To obtain a larger quantitative effect in pituitary tumor growth for F344 than that given in Table 1, we extended the DES treatment period in subsequent experiments to 10 weeks because pituitary mass increases continuously with time from administration of the implant for at least 12 weeks (10). We also switched to using females exclusively because F344 females produce tumors twice as massive as males under the same treatment (12). Ten weeks of chronic treatment with DES induced hemorrhagic pituitary tumors in female F344 rats that had, on average, 10 times normal mass (Table 2). All of the 25 DES-treated F344 females had hemorrhagic tumors typified by the one shown in Fig. 1. To obtain a quantitative index for development of a hemorrhagic pituitary, we assayed hemoglobin, because this reflects the quantity of red blood cells in the organ, and found that pituitaries of DES-treated F344 rats had 6-fold greater hemoglobin concentration than untreated (Fig. 2).

We have found that the hemorrhagic appearance is 100% penetrant in pituitaries of female F344 rats treated with DES for at least 8 weeks. If shorter treatment periods (3 to 6 weeks) are used, not all rats produce hemorrhagic pituitaries, particularly in males. Such a result was observed by DeNicola *et al.* (6) who reported that some, but not all, pituitaries of male F344 rats after 60 days of DES treatment were hemorrhagic. Their histological examination showed that both groups of DES-treated F344 rats exhibited hyperplasia and hypertrophy of the lactotrophs, but those that were hemorrhagic also had hemorrhagic lakes (6).

Table 1. Effect of DES treatment on pituitary mass in male rats of different strains

	Linear scale (mg)					Log scale (log of mg)				
	Untreated		8 week DES			Untreated		8 week DES		
Strain	*n*	Mean (SD)	*n*	Mean (SD)	*P* value*	*n*	Mean (SD)	*n*	Mean (SD)	*P* value*
Holtzman	5	12.3 (1.5)	10	15.5 (4.9)	$0.05 > P > 0.025$	5	1.09 (0.05)	10	1.17 (0.14)	$P \approx 0.05$
F344	5	8.8 (1.0)	10	31.5 (14.6)	$0.001 > P$	5	0.94 (0.05)	10	1.44 (0.25)	$0.001 > P$
BN	5	7.7 (1.0)	10	8.1 (1.2)	$0.10 > P > 0.05$	5	0.88 (0.05)	10	0.90 (0.07)	$P > 0.10$
Buffalo	5	11.1 (3.3)	9	12.6 (3.0)	$0.10 > P > 0.05$	5	1.03 (0.15)	9	1.09 (0.11)	$0.10 > P > 0.05$
Copenhagen 2331	7	7.3 (1.1)	9	11.6 (5.6)	$P \approx 0.05$	7	0.89 (0.06)	9	1.02 (0.22)	$0.05 > P > 0.025$
Wistar Kyoto	6	6.9 (1.7)	10	7.3 (2.5)	$P > 0.10$	6	0.83 (0.13)	10	0.85 (0.12)	$P > 0.10$

*P value of one-sided t test of DES-treated versus untreated rats. H_0, no difference; H_A, DES-treated > untreated.

With the same 10-week DES treatment period, BN rats did not exhibit an increase in pituitary mass (Table 2), hemorrhagic appearance (Fig. 1), or increased hemoglobin concentration (Fig. 2*A*). DES treatment caused a small increase in pituitary DNA content, but it was negligible compared with the increase in DNA in the DES-treated F344 pituitary (Fig. 2*B*). We observed no increase in mass even if BN rats were treated for 12 weeks (data not shown).

Expression of Growth and Hemorrhagic Phenotypes in an F_1 Hybrid. We expected that if pituitary tumor formation was expressed by an F344 × BN F_1 hybrid, the F_1 generation would also express the hemorrhagic phenotype. While the pituitaries of DES-treated F_1 rats underwent significant growth to become 3-fold or 5 standard deviations greater in mass than untreated rats (Table 2), they did not exhibit the hemorrhagic phenotype of F344 (Fig. 1) or increase in hemoglobin concentration (Fig. 2*A*). In fact, F_1 hemoglobin levels were identical to those of BN (Fig. 2). In all 29 F_1 females treated with DES for 10 weeks, outward morphology was normal, except for greatly enlarged anterior lobes (Fig. 1). Even if the treatment of the F_1 were continued for up to 14 weeks, outward morphology did not change, though their pituitaries continued to grow for at least 12 weeks (data not shown). Pituitary DNA content (Fig. 2*B*) followed the same pattern as total mass, indicating that the increase in mass reflects cell proliferation, consistent with earlier studies (12).

Other authors have defined pituitary tumor formation based on reaching some critical mass threshold (12, 20). However, given that this phenomenon is a dispersed hyperplasia not originating from a single clone (4) and that our control strain, BN, exhibits no statistically significant increase in pituitary mass (Tables 1 and 2), we consider any significant increase in mass to be biologically relevant.

Segregation of Growth and Hemorrhagic Phenotypes in the F_2 Generation. Since the F_1 generation's tumor phenotype suggested separability of growth and hemorrhagic phenotypes, we performed an F_2 intercross to determine if the traits segregated independently. We found a significant degree of independence: only 5 out of 160 DES-treated F_2 rats had hemorrhagic pituitaries, whereas most of the 160 rats had tumor mass significantly greater than DES-treated BN (Fig. 3*B*). However, independence was not complete as the five hemorrhagic pituitaries were all among the very largest (Fig. 3*B*). This raised the question as to whether the hemorrhagic appearance could be a by-product of extensive growth. However, comparison of F344 tumors to all F_2 tumors in a comparable range of mass proved otherwise. Pituitary mass of F344 females after 10 weeks of DES treatment ranged as low as 40 mg, or 1.60 on a log scale (Fig. 3*A*), but, regardless of mass, all F344 pituitaries exhibited the hemorrhagic phenotype after 10 weeks of DES treatment. Conversely, 36 of the DES-treated F_2 rats (23% of the population) formed pituitary tumors that were above 40 mg (Fig. 3*B*), but 31 of these were clearly not hemorrhagic. We assayed hemoglobin content of the 24 largest F_2 pituitaries and found that all F_2 pituitaries that we visually scored as hemorrhagic also had elevated hemoglobin concentration that was significantly greater than that of the DES-treated F_1 hybrid ($P < 0.001$). As with the F_1 hybrid, nonhemorrhagic F_2 tumors had the hemoglobin concentration of the DES-treated BN (Fig. 3*C*). Two of the F_2 pituitaries that we visually scored as hemorrhagic were not as drastically hemorrhagic as the F344 control, but had hemorrhagic sections (data not shown). Variation in hemorrhagic phenotype is also reflected in hemoglobin content as some of the hemorrhagic F_2 did not have hemoglobin levels as high as the F344 average, although all of their levels were still greater than the nonhemorrhagic F_1 pituitary. The quantitative hemoglobin values did seem to still fall into two groups of F_1-like and significantly elevated (Fig. 3*C*).

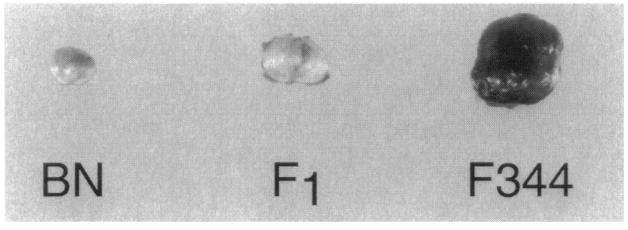

FIG. 1. Strain-dependent effects of DES treatment on rat pituitaries. Representative pituitaries of DES-treated BN, F344, and their F_1 hybrid.

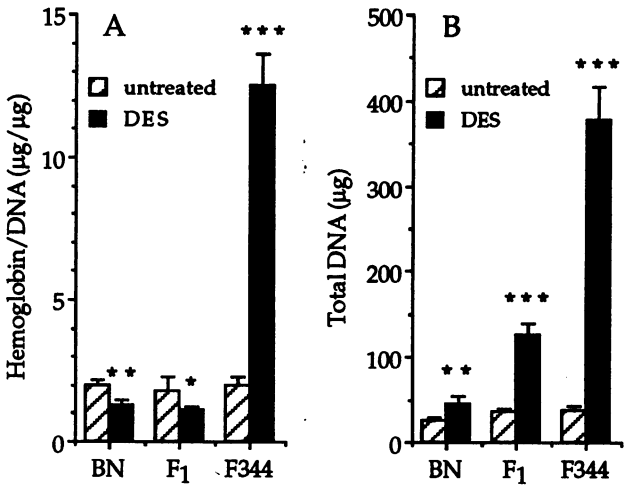

FIG. 2. Effect of DES treatment on rat pituitary DNA and hemoglobin content. (*A*) Pituitary hemoglobin concentration, expressed as hemoglobin/DNA ratio. (*B*) Pituitary DNA content. DES-treated is average of six individuals and untreated is average of four individuals. Error bars are one standard error. One-sided *t* test *P* values are indicated by the asterisks above the DES-treated bar; *, $0.10 > P > 0.05$ (not statistically significant); **, $0.05 > P > 0.010$ (moderately significant); ***, $P < 0.001$ (highly significant).

DISCUSSION

In the estrogen-dependent rat pituitary tumor, we have used simple genetic crosses to separate growth in mass from the morphological changes of the hemorrhagic phenotype. When the F344 strain was crossed with the tumor-resistant BN strain, their F_1 progeny exhibited tumor growth, but not the hemorrhagic phenotype. In progeny of an F_2 intercross, where the multiple genes responsible for development of the F344 tumor are recombined, pituitaries as large as 10 times normal mass were produced that were not hemorrhagic (Fig. 3*B*). Not only did the DES-treated F_1 and most of the DES-treated F_2 offspring lack hemorrhagic development, their hemoglobin content was identical to the tumor-resistant BN strain. Thus, they displayed no increase in red blood cell concentration, despite their significant increase in mass.

The failure to detect changes in hemoglobin content in the F_1 hybrid indicates that some general angiogenesis accompanies tumor growth, but it is only proportional to the added tissue mass. However, a significant quantitative increase in the blood supply to the tumors, as would be measured by hemoglobin content, does not appear to drive tumor formation.

From the phenotypes of the F_1 hybrid, we conclude that the hemorrhagic phenotype is a recessive trait, and from the low frequency in the F_2 generation, we conclude that it is due to multiple genes with epistasis (Table 3). The observed frequency is between that predicted by two-locus and three-locus models and neither can be rejected by χ^2 test (21). We have found variation in the degree of hemorrhagic phenotype; some

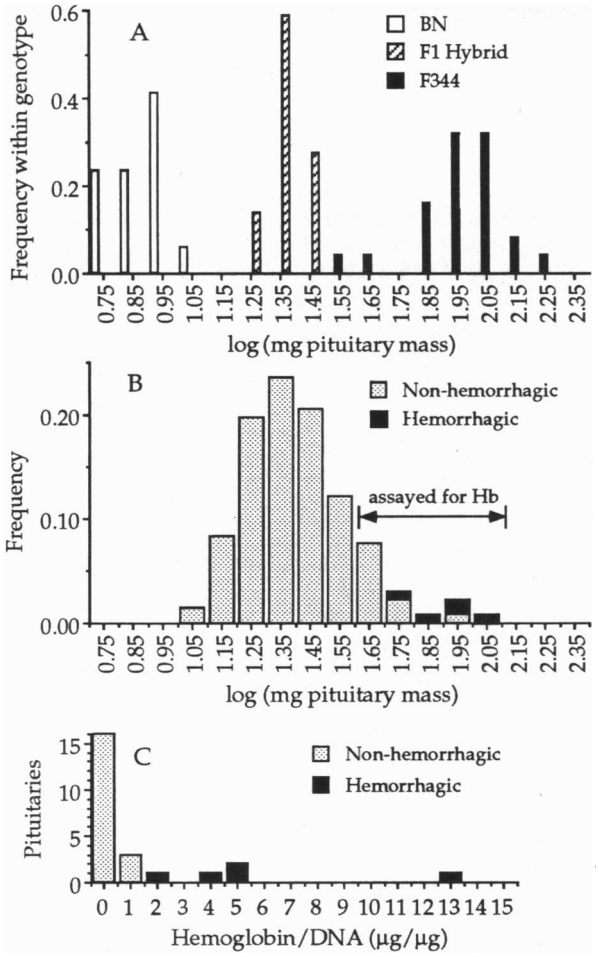

FIG. 3. Inheritance of tumor phenotypes in progeny of crosses of F344 and BN. (*A*) Frequency distribution of pituitary mass in 10 weeks DES-treated female BN, F344, and their F_1 hybrid. (*B*) Frequency distribution of pituitary tumor phenotypes in 160 DES-treated F_2 progeny. The dark shaded portion of each bar is the proportion of the mass class that displayed the hemorrhagic phenotype of the DES-treated F344 grandparental line shown in Fig. 1. (*C*) Pituitary hemoglobin, expressed as hemoglobin/DNA ratio, of the 24 largest F_2 pituitaries (indicated by the arrow in *B*).

of the hemorrhagic F_2 pituitaries were not as extreme as the F344 strain. Thus, a purely qualitative view of the inheritance of the hemorrhagic phenotype may not be perfect.

Overall pituitary growth response to estrogen is an additive trait. Both the DES-treated F_1 and F_2 generations had a mean pituitary mass intermediate between BN and F344 (Table 2). The broad, continuous distribution of the F_2 generation (Fig. 3) also leads us to view tumor mass as an additive polygenic growth trait. If variation in mass was due to a single gene, the F_2 would reconstitute the BN, F_1, and F344 masses in a 1:2:1 ratio, which would have been detectable because the phenotypes of those three groups are completely separable after 10 weeks of DES treatment (Fig. 3). Our working hypothesis is a modification of a model proposed by Wiklund *et al.* (12). They classified pituitaries in F_2 intercross and backcross populations as tumors if they exceeded a threshold of 17.5 mg for males and 19 mg for females and predicted that two or three genes were responsible for the estrogen-dependent tumor formation (12). In contrast, our quantitative "growth trait" model views all variation in mass as physiologically significant. For example, under a quantitative model, a 50 mg and 25 mg pituitary are viewed as different, whereas under the tumor incidence model of Wiklund *et al.* (12) they are classified as the same. A quantitative view was also suggested by Holtzman *et al.* (11), who compared the effect of DES treatment on ACI and Sprague–Dawley rats and stated that the tumor formation in ACI may reflect quantitative differences in response, rather than absolute susceptibility.

Table 3. Genetic models for inheritance of hemorrhagic tumors

Loci	Hemorrhagic genotype	Expected hemorrhagic F_2	χ^{2*}	*P* value*
1	aa	40.0	40.8	<0.001
2	aa bb	10.0	2.7	0.100
3	aa bb cc	2.5	2.9	0.100
3	aa bb C_	7.5	0.9	>0.250
4	aa bb cc dd	0.6	16.6	<0.001

Test for goodness of fit of simple genetic models for inheritance of hemorrhagic phenotype. Each letter is a different locus and lowercase letters denote recessive (F344) allele.

*χ^2 test statistic for the deviation of the observed frequency of 5 hemorrhagic and 155 nonhemorrhagic from that expected by the given genetic models; 1 degree of freedom. H_0, deviation from expected frequency is due to random chance.

Because we take a different view of pituitary tumor formation than was used to form the three-locus model of Wiklund *et al.* (12), we should reexamine gene number. Using Lande's (22) estimator for the minimum effective number of loci affecting a quantitative trait, we get an estimate of at least five loci. However, such an estimation is built with many necessary simplifying assumptions, such as pure additivity and all loci having equal effects (22). All that we can conclude with surety is that there is more than one locus, but not a huge number of loci such as 10 or more, as is seen with many agronomic traits (23).

Though the two phenotypes are expressed differently, they must share, in part, a common genetic basis because only the most massive F_2 tumors were hemorrhagic (Fig. 3 *A* and *B*). Our results can be explained by a genetic model with several genes encoding components of an estrogen signal transduction pathway that regulates pituitary growth and development. We suggest that two or three of these genes regulate both lactotroph proliferation and tissue rearrangement, although there may be additional loci regulating growth only. For each gene that regulates both phenotypes, one BN allele is sufficient to give control of angiogenesis, but not sufficient to suppress growth. Thus, F_1 hybrid and most F_2 rats do not exhibit the hemorrhagic phenotype. The difference between nonhemorrhagic and hemorrhagic rat pituitaries may reflect differences in the regulation of proteases involved in tissue degradation and rearrangement (2, 3, 9). Analysis of the genes directing expression of the growth and hemorrhagic phenotypes promises to reveal important branch points in regulatory pathways of tumor growth and development.

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